Review

Boundaries that demarcate structural and functional domains of chromatin

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Understanding of how the eukaryotic genome is packaged into chromatin and what the functional consequences of this organization are has begun to emerge recently. The concept of 'chromatin domains' – the topologically independent structural unit – is the basis of higher order chromatin organization. The idea that this structural unit may also coincide with the functional unit, offers a useful framework in dissecting the structure–function relationship. Boundaries that define these domains have been identified and several assays have been developed to test them *in vivo*. We have used genetic means to identify and analyse such boundary elements in the bithorax complex of *Drosophila melanogaster*. In this review we discuss chromatin domain boundaries identified in several systems using different means. Although there is no significant sequence conservation among various chromatin domain boundaries, these elements show functional conservation across the species. Finally, we discuss mechanistic aspects of how chromatin domain boundaries may function in organizing and regulating eukaryotic genome.

1. Structural and functional compartmentalization of the nucleus

The genome of eukaryotes must be extremely compacted in order to fit in the nucleus. How this compact structure can still function as substrate for complex processes like transcription and replication remains mostly unknown. Similarly, how these processes and others like RNA processing/transport, import and sub nuclear localization of proteins, genome packaging and cell division are integrated into nuclear structure is unclear. Even half a century after the discovery of DNA structure, we remain ignorant of many important aspects of how this genetic material is used in a eukaryotic nucleus. This is largely due to the fact that we do not know how genes are organized and packed in the nucleus. The high concentration of large DNA threads (for example, 7 mg/ml of DNA in the nucleus of a human cell) implies that the nuclear environment is very viscous and, therefore, in order to avoid energy cost of the molecular movements any traffic has to be well controlled within the context of nuclear architecture and chromatin organization.

Chromosome specific and gene specific fluorescent probes have been used to investigate nuclear architecture *in situ*. Such studies have demonstrated that individual chromosomes have their own territories in the interphase nucleus, which may play a role in the functional compartmentalization of the nucleus (Manuelidis 1985; Manuelidis and Borden 1988; Ferreira *et al* 1997; Misteli and Spector 1998; Zink *et al* 1998). Figure 1 is a model of the nucleus of human cell in which individual chromosomes are seen to have distinct territories. Similar chromosomal territories have been observed in several cell types of different organisms. However, it remains to be established if such an organization is universal or widespread. While such studies provide a global picture of the nucleus, they do not suggest what might be the structural basis of such an organization. Also, relative positioning of the chromosomes, if any, its dynamics and interaction of individual territories with the nuclear membrane are not understood. Within their chromosomal territory genes are situated preferably at the periphery and the non-coding DNA appears to be packed into the interior of the territory (Kurz *et al* 1996). Furthermore,

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replication, transcription and pre-mRNA processing appear to take place at distinct and discrete sites (Spector 1993; Misteli and Spector 1998). From the localization of RNA foci and tracks (Lawrence *et al* 1989; Xing and Lawrence 1991; Rosbash and Singer 1993), it appears that not only various nuclear processes take place in specified compartments, the finished products are transported along a defined path and not by free diffusion. It appears, therefore, that the nuclear interior is a well organized and regulated place. The structural components and the regulatory mechanism involved in these processes are not clear.

While a variety of evidence support the functional compartmentalization of the interphase nucleus, the structural basis for such organization remains to be established. Nuclear matrix or chromosomal scaffold has been implicated as a proteinaceous nuclear skeleton that provides a framework for the organization of chromatin. Although many studies on nuclear matrix have been disputed, at least some of the careful analyses carried out

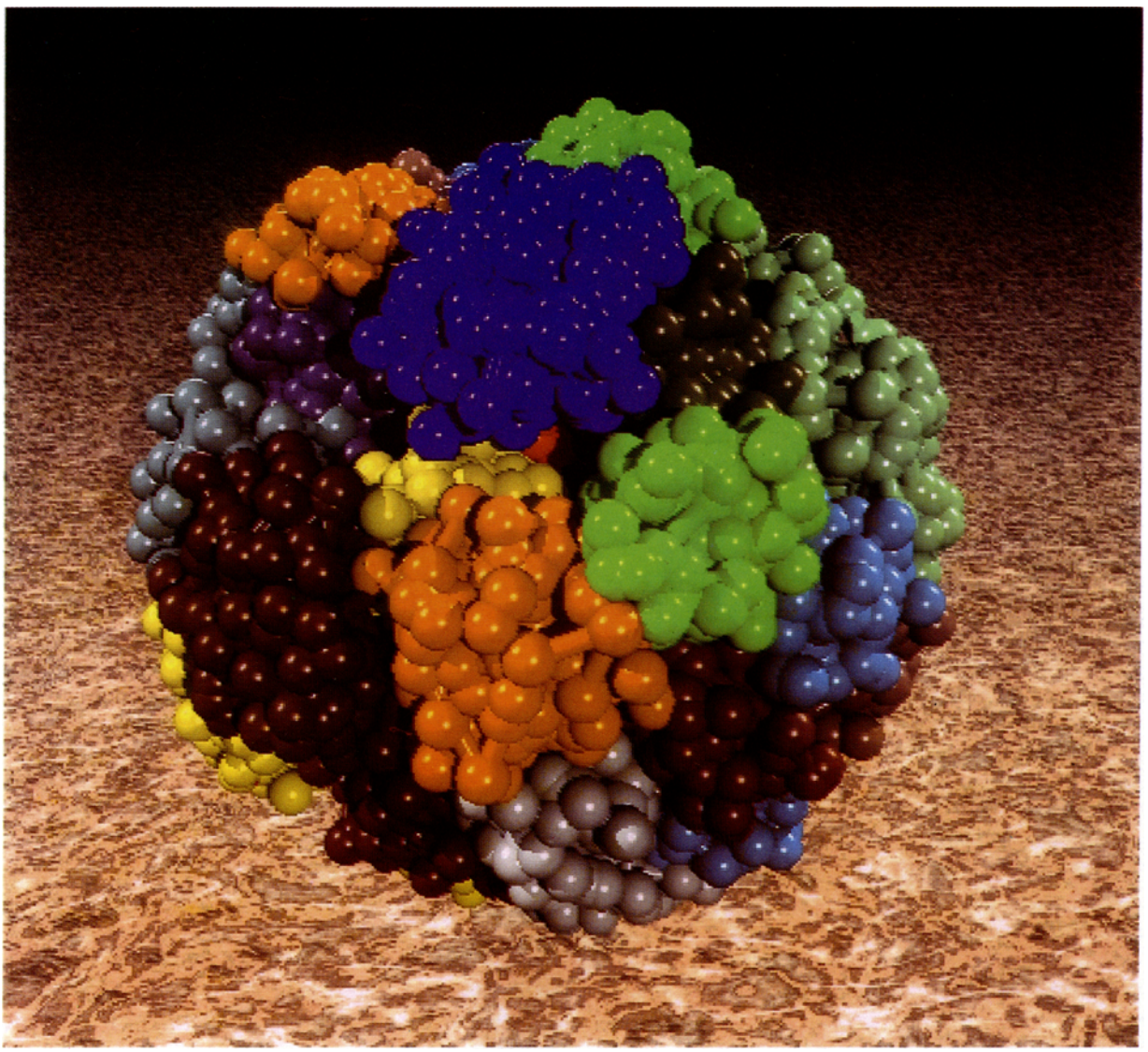

Figure 1. A model of spherical human cell nucleus. The individual chromosome territories shown in different colours are assumed to consist of individual, mutually exclusive chromatin granules (corresponding to replication foci or R/G band) with a DNA content of 1 Mbp each and a diameter of 500 nm. The late replicating G-band domains and the early replicating R-band domains are assumed to be formed by loop clusters of about 120 kbp each. For the simplicity of calculation, the 1 Mbp-granules were modeled as spheres. To account for the observed dynamic variability in the distances between chromatin granule domains/replication domains, zigzag linkers with random angles were introduced. This model makes no assumption on the chromatin topology inside the R/G domains. (G Kreth, Ch. Muenkel, C Cremer, unpublished results. Picture kindly provided by Prof. C Cremer).

with least manipulation and under physiological conditions have begun to improve our understanding of the link between structural and functional subdivision of chromatin into distinct domains (see below). We are only beginning to understand what kind of matrix provides the frame work for the nuclear architecture and what proteins and DNA elements are involved in it.

2. Organization of DNA in the nucleus

In a human cell 1·6 meter long DNA is packaged in a nucleus of 5 micrometer diameter. Packaging so much of DNA in such a small space and in a form that accommodates replication and transcription is a feat that every eukaryotic cell performs every time it divides. Table 1 gives an outline of how chromosomes are organized in the nucleus. In this organization, the structure beyond the 30 nm fiber – the so called higher order chromatin organization – is not entirely clear. A variety of cytological and biochemical studies have, however, led to the conclusion that the interphase chromatin is organized into topologically distinct domains of varying sizes. Here we review the work related to the chromatin domain with particular emphasis on the boundaries of such domains and present the argument that the structurally defined chromatin domains may coincide with the functional domains of gene regulation. We also discuss work from our laboratory in which first genetic evidence for the existence of such domain boundaries was provided and their regulatory function was demonstrated *in vivo*.

2.1 *The eukaryotic genome is organized into topologically independent chromatin domains*

It was evident from the early electron microscopic studies that chromatin domains are formed by the looping of the 30 nm fiber along the chromosomal scaffold of mitotic chromosome. Biochemical and biophysical approaches have been used subsequently to investigate if the cytologically invisible interphase chromatin is also organized into domains of similar kind. Table 2 summarizes studies wherein various techniques have been employed to investigate if interphase chromatin is organized in domains and if so, the average size of such domains. While it is clear that most of the genome in different organisms is organized in the form of domains, there is quite a variation in the estimate of average size of domains in the nuclei from different sources or by use of different techniques on the nuclei from one source. These apparent discrepancies can be explained by assuming that domain size may vary in different organisms or different tissues of the same organism. Also, some of the domains may not be stable enough to withstand the experimental condition and merge with the neighbouring domains or collapse on the nuclear skeleton and get subdivided into smaller loops. It is generally believed that the eukaryotic genome is organized in 5–200 kb domains. It is not clear though if average domain size has any correlation with the genome size or content of repetitive DNA. Table 3 summarizes the size of particular domains in which a gene or group of genes reside. At least in some cases different methods give similar size estimates. It is often observed that highly expressed genes tend to reside in smaller domains of 4–13 kb (Gasser and Laemmli 1987). These findings suggest that packaging of genome into domains may have functional consequences.

2.2 *Functional domains of gene regulation*

Chromosomal rearrangements, in which the coding regions and regulatory elements remain intact, can occasionally result in a mutant phenotype. Position effect variegation

Table 1. Packaging of DNA in chromatin[a].

Structure	Size	DNA/unit	Compaction	Comments
Double helix	2 nm	10·4 bp	–	–
Nucleosome	10 nm	146 bp	6-fold	DNA wraps almost twice around a core histone octamer
Chromatin fiber	30 nm	900 bp or 6 nucleosomes	40-fold	Histone H1 or a related proteins binds the 40–70 bp linker DNA[b] that separates adjacent core particles of 'beads-on-a-string'
Chromatin loop or domain	(see tables 2 and 3)	180–300 nucleosomes	700-fold	The 30 nm fiber forms topologically independent units
Chromomere	800–1000 nm	15–18 loops	10000-fold	In case of metaphase chromosome, adjacent loop attachment sites are arranged in helical spiral along the long axis of the metaphase scaffold

[a]This table outlines the chromatin organization present in most cell types. However, entirely different kind of chromatin organization can be found in specific cell types, for example in spermatozoa.
[b]The length of the linker DNA may vary significantly due to positioning of nucleosome by as yet unknown sequence elements and such nucleosome free sites may form crucial regulatory elements.

(PEV) in *Drosophila* is one such example which has been studied most extensively and is thought to be a consequence of a transcribed region being transposed in the vicinity of transcriptionally inert heterochromatin (Reuter and Spierer 1992). This suggests that the local chromosomal context has an influence on the transcription of a resident gene. That gene expression is context dependent is directly demonstrated by transgene experiments in a variety of model organisms. Frequently, independent transgenic lines show variation in expression of the reporter gene that they carry (Wilson *et al* 1990). This variation is attributed to the fact that in different lines the transgene occupies a different environment of enhancers or silencers which influences its expression. Why enhancers and silencers, therefore, do not misregulate genes in their native context? The most attractive model suggests that genes and their regulatory elements are confined in functionally distinct domains defined by the boundary elements.

3. The boundaries of the chromatin domains

The concept that higher order chromatin organization begins with chromatin domains, the topologically independent structural unit, has streamlined studies to understand how eukaryotic genome is packaged into chromatin and what are the functional consequences of this organization. The organization of eukaryotic genome in functional and structural domains necessarily implicates the existence of boundaries of such domains. Several assays have been developed recently and used successfully in a variety of systems to isolate and test such boundary elements (see below).

One of the important issues with respect to the boundary elements is whether or not structural and functional boundaries are the same. The idea that structural units may also coincide with the functional units, offers a useful framework in dissecting this structure–function relationship to understand possible regulatory mechanisms at the level of chromatin organization.

3.1 *Assays for chromatin domain boundaries*

The following assays are based on different properties that are expected from the boundary elements. The first two assays (3.1a, b) are based on structural features while the other two (3.1c, d) are based on functional aspects of a putative boundary. All the boundaries may not have similar properties or mechanism of function and hence may respond differently to different assays. While each of these assays is useful in identifying putative boundary elements or studying such elements in a defined region of

Table 2. Average size of chromatin domain/loops.

Chromatin source	Domain size (kb)	Method	Reference
Yeast	< 250	Sedimentation, analysis of fragments	Pinon and Salts 1977
Maize	45	DNase I and endogenous nuclease digestion, analysis of fragments	Paul and Ferl 1998
	50	Topoisomersase II mediated cleavage, nuclease digestion, analysis of fragments	Espinas and Carballo 1993
Lily	35	DNase I digestion, analysis of fragments	Paul and Ferl 1998
Arabidopsis	25	DNase I digestion, analysis of fragments	Paul and Ferl 1998
Drosophila	85	Nicking and change in sedimentation coefficient	Benyajati and Worcel 1976
Chicken/erythrocyte	45	Nuclease digestion, analysis of fragments	Ganguly *et al* 1983
	40	Nuclease digestion, analysis of fragments	Hyde 1982
Mouse/3T3	90	Dimensions of 'halo' in nucleoids	Vogelstein *et al* 1980
Mouse/P815	53	EM on nucleoids	Hancock and Boulikas 1982
Mouse/liver	62	Digestion, sedimentation	Razin *et al* 1979
Rat/liver	35	Nuclease digestion, analysis of fragments	Igo-Kemenes *et al* 1977
	80	Estimate of number of loops per genome	Berezney and Buchholtz 1981
	50	Nuclease digestion, analysis of fragments	Filipski *et al* 1990
Rat/thymocyte	50	Topoisomersase II mediated cleavage, analysis of fragments	Filipski *et al* 1990
Human/HeLa cell	43	EM on histone depleted metaphase chromosomes	Paulson and Laemmli 1977
	83	EM on histone depleted metaphase chromosomes	Earnshaw and Laemmli 1983
	220	Fluorimetry (ethidium binding)	Cook and Brazell 1978
	86 (50–175)	Nuclease digestion, analysis of fragments	Jackson *et al* 1990b
	12 and 50–250	Fluorimetry (ethidium binding)	Jackson *et al* 1990b
Human/epithelial tissue	50	Apoptosis induced cleavage, analysis of fragments	Oberhammer *et al* 1993

genome, characterization of any boundary remains tentative unless it has been tested to meet several of the criteria that form the basis of different assays. Also, the choice of tissue used for an assay may be critical as all the boundaries may not be functioning in all the tissues all the time.

3.1a *The MAR or SAR assay*: Matrix associated region (MAR) or scaffold associated region (SAR) assay is based on the structural basis of boundary elements which are expected to be attached to the nuclear-skeleton in order to create topologically independent domains or

Table 3. Domain size for specific gene.

Organism/gene	Domain size (kb)	Method	Reference
Yeast			
HMR locus	3·5	Mapping of flanking boundaries	Donze *et al* 1999
Maize			
Adh	90	Topoisomerase II mediated cleavage and DNase I digestion	Paul and Ferl 1998
GRF1	100	Topoisomerase II mediated cleavage and DNase I digestion	Paul and Ferl 1998
Arabidopsis			
Adh	8·3 and 6·1	Topoisomerase II mediated cleavage and DNase I digestion	Paul and Ferl 1998
GRF	27	Topoisomerase II mediated cleavage and DNase I digestion	Paul and Ferl 1998
hmg-like and PC Sterol-methyltransferase and Thioredoxin-like	5 5	Localization of flanking MARs Localization of flanking MARs	van Drunen *et al* 1997 van Drunen *et al* 1997
Tomato			
HSC80	8	Topoisomerase II mediated cleavage	Chinn and Comai 1996
Bean			
β-phaseolin	3·3	Topoisomerase II mediated cleavage	van der Geest *et al* 1994
Rice			
Sh2	6·6	Localization of flanking MARs	Avramova *et al* 1998
X	10·8	Localization of flanking MARs	Avramova *et al* 1998
A1	≥ 8·9	Localization of flanking MARs	Avramova *et al* 1998
Sorghum			
Sh2	7·2	Localization of flanking MARs	Avramova *et al* 1998
X	16·3	Localization of flanking MARs	Avramova *et al* 1998
A1-a	9·2	Localization of flanking MARs	Avramova *et al* 1998
A1-b	8·3	Localization of flanking MARs	Avramova *et al* 1998
Drosophila			
HSP70	15	Mapping of SCS and SCS′ elements	Udvardy *et al* 1985
Histone genes	5	Localization of flanking MARs	Mirkovitch *et al* 1984
S8	43	Localization of flanking MARs	Mirkovitch *et al* 1986
B16-I and C9a	112	Localization of flanking MARs	Mirkovitch *et al* 1986
Ace	26	Localization of flanking MARs	Mirkovitch *et al* 1986
Adh	5	Localization of flanking MARs	Gasser and Laemmli 1986
Sgs-4	4·7	Localization of flanking MARs	Gasser and Laemmli 1986
ftz	11·1	Localization of flanking MARs	Gasser and Laemmli 1986
X chromosome	15–115	Localization of flanking MARs	Surdej *et al* 1990
(14B–15B region)	50–90	Topoisomerase II mediated cleavage	Iarovaia *et al* 1996
Chicken			
Ovalbumin	100	DNase I digestion	Lawson *et al* 1982
Lysozyme	24	DNase I digestion Localization of flanking MARs	Jantzen *et al* 1986 Loc and Stratling 1988
β-globin	33	DNase I digestion and chromatin immuno-precipitation with Ac-histone antibodies	Hebbes *et al* 1994
Human			
C-myc	80–100	DNase I digestion Topoisomerase II mediated cleavage	Gromova *et al* 1995a Gromova *et al* 1995b
rDNA	45	Topoisomerase II mediated cleavage and Bal31 digestion	Iarovaia *et al* 1995
Apo B	47·5	DNase I digestion	Levy-Wilson and Fortier 1989

loops. There are two complementary approaches to isolate MARs. One approach is to treat isolated nuclei with mild detergent to remove histones and cleave off the genomic DNA with restriction endonuclease. After these treatments, the DNA still bound to the matrix, the *in vivo* MAR, is isolated and analysed (Mirkovitch *et al* 1984). In the second method, histone depleted nucleus is incubated with DNase I to degrade DNA completely and the remaining proteinaceous structure, the nuclear matrix, is used to test *in vitro* if a given DNA sequence can bind to it which will tell if the sequence contains a MAR or not (Izaurralde *et al* 1988). MAR/SAR assays identify DNA sequences that are associated with nuclear matrix, which by itself does not prove that they function as boundaries. On the other hand, it is not entirely unlikely that some boundaries may function without associating with matrix or their association with matrix may be transient or unstable under the experimental conditions.

3.1b *DNase I hypersensitivity and topoisomerase II cleavage assays*: A mild treatment with DNase I is frequently used to investigate the accessibility of a given region of genome. There are two kinds of DNase I hypersensitivities. A general DNase I hypersensitivity corresponding to transcriptionally active region is about 10-fold more sensitive as compared to the transcriptionally inactive region and is spread over several kilo bases (Weintraub and Groudine 1976). The second kind of DNase I hypersensitivity is marked by 100-fold or more accessibility to cleavage and usually involves 100–200 bp segments of DNA (Wu *et al* 1979). These hypersensitive sites generally denote DNA sequence of special function. This assay, although not exclusively used for boundary analysis, is extremely useful in molecular mapping of a boundary element in a suspected region of genome. A boundary structure is expected to be formed with the help of a group of proteins and the DNA should, therefore, remain nucleosome free. This and the close proximity of boundary sequences to the nuclear matrix should give rise to a defined set of hypersensitive sites. Once such sites are mapped, the boundary properties of a defined sequence can be tested using other assays.

Topoisomerase II is one of the components of the nuclear skeleton where the base of the chromatin loop is secured. It is proposed that this protein is a structural component of the base of the chromatin loop bound to the nuclear skeleton and modulates the topology of the loop from there. Several inhibitors of topoisomerase II allow the cleavage but block the religation activity of the enzyme and hence generate DNA cleavage at topoisomerase II binding sites. Such drugs have been used to study chromatin domain organizations (Cockerill and Garrard 1986; Kas and Laemmli 1992). Since majority of the MARs have topoisomerase II binding sites, this assay may also be considered as one of the ways to map MARs.

3.1c *Position effect and enhancer blocking assays*: Expression of transgenes is known to be influenced by the regulatory environment of the insertion site in the genome. If the construct carrying the reporter gene is designed in such a way that the coding region and the regulatory sequences are flanked by boundary elements the expression should no longer be influenced by the local chromatin environment (figure 2A). This will translate into a position independent and copy number dependent expression of the reporter gene driven by the regulatory elements included within the construct (Kellum and Schedl 1991). This assay, referred to as the position-effect assay, provides a means to test if a given DNA sequence can create an independent domain for the transgene at the site of insertion.

Enhancer blocking assay is a modification of the position effect assay where the DNA fragment to be tested for the boundary function is placed between the promoter and the enhancer elements of the transgene construct (figure 2B) (Kellum and Schedl 1992). A boundary element should block the enhancer from acting on a promoter if situated between the two, whereas control DNA fragment of similar size should not affect the enhancer promoter interaction ruling out a simple distance effect. In recent versions of this assay, two reporter genes are used, one to score the transformed cell or organism and the other to test the enhancer blocking activity.

3.1d *Genetic or phenotypic assays*: This is a rather special but biologically most relevant assay. In a situation where two adjacent domains of distinguishable activities exist, a boundary element is expected to separate the two domains. If this boundary is mutated the two domains will fuse to form a novel domain and, therefore, lose their wild type properties. In certain circumstances such boundary deletions may be revealed by a phenotype. This is the case in the bithorax complex (BX-C) of *Drosophila* where such mutations have led to the identification of chromatin domain boundaries that subdivide the complex into independent regulatory domains, see § 4 (Gyurkovics *et al* 1990; Mihaly *et al* 1998a). So far, no point mutation with an associated boundary phenotype has been isolated.

3.2 *Major chromatin domain boundaries*

Many boundary elements have been identified and analysed using the assays described above (see table 4). We summarize the main features of these elements and discuss the possibility that some of these may meet both structural and functional criteria of such elements. This is particularly significant when a boundary identified in one assay has been successfully tested in another assay.

3.2a *MAR/SAR*: MARs are 300–2000 bp long with the core activity spread over 200–1000 bp. A variety of

modules viz., origin of replication, topoisomerase II binding sites, TG rich sequences, curved DNA, kinked DNA, A- and T-boxes, ATATTT motif, poly A or T tracts etc., cluster together to constitute a MAR. In a recent theoretical study Singh *et al* (1997) used such criteria to develop a mathematical model to predict MARs in a stretch of DNA sequence. Interestingly, this method could identify several experimentally identified MARs.

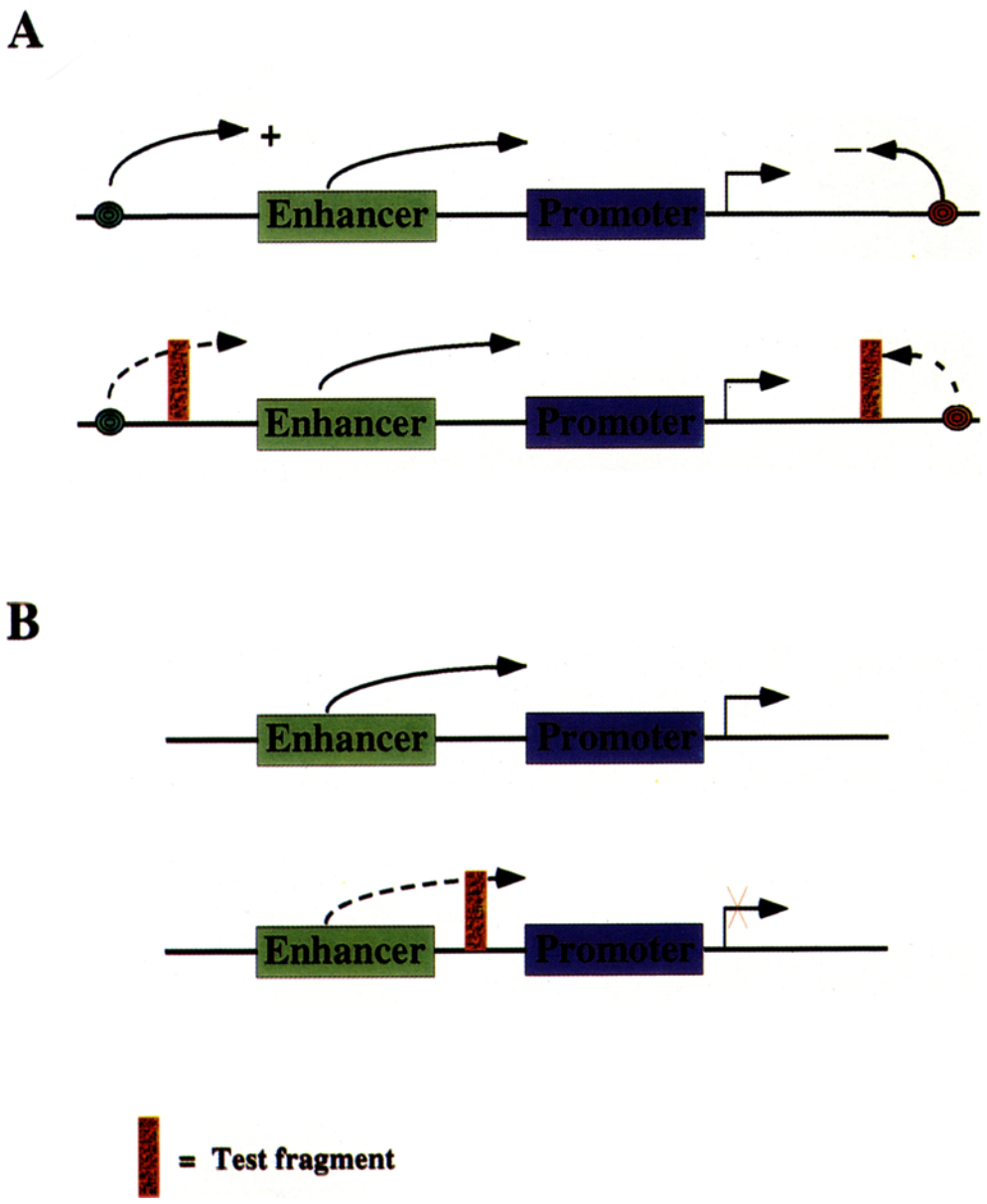

Figure 2. Assays for chromatin domain boundaries. Curved arrows show the interaction of indicated regulatory elements. Broken lines indicate that if the test fragment, shown as red block, is a boundary element as such interactions are weakend or eliminated. (**A**) Insulation from position effect – usually different lines of the transgenic organism show varied expression levels due to the contribution of positive and negative regulatory elements (shown as green and red circles, respectively) near the site of insertion. When boundary DNA is added on both sides of the reporter gene, it can prevent both the positive and negative influences. In the absence of such position effects, indicated by the broken arrows, several transgene lines give similar level of reporter gene expression. (**B**) Enhancer blocking assay – the transgene construct carrying the reporter gene driven by the shown enhancer and promoter will decrease the expression of the reporter gene when a boundary element is interposed between the promoter and enhancer.

Table 4. Chromatin domain boundary elements.

Boundary	Location	Assay	Reference
MARs			
Soybean	3′ region of heat shock gene Gmhsp 17·6-L	Insulation from PE in transgenic tobacco plants	Schoffl *et al* 1993
Bean	β-phaseolin gene	Insulation from PE in tobacco	van der Geest 1994
Tobacco	(not mapped)	Insulation from PE in tobacco	Breyne *et al* 1992
Chicken	Within lysozyme LCR	Insulation from PE in rat fibroblasts	Phi-Van *et al* 1990
		Insulation from PE in mouse 3T3 cells	Phi-Van and Stratling 1996
		Insulation from PE in transgenic mouse	Lee *et al* 1998
		Insulation from PE in transgenic mouse	McKnight *et al* 1992, 1996
Human	Apolipoprotein B and α1-antitrypsin loci	Insulation from PE in hepatoma cells	Kalos and Fournier 1995
		Insulation from PE in *Drosophila*	Namciu *et al* 1998
LCRs			
β-globin locus of chicken	DNase I hypersensitive site 4 (5′HS4)	Enhancer blocker in human erythroleukemic line K562 and insulator from PE in *Drosophila*	Chung *et al* 1993
Lysozyme locus of chicken	Element A	Insulation from PE in transgenic mouse	Bonifer *et al* 1990; Bonifer *et al* 1994
Metallothionein locus of mouse	Flanking sequences of metallothionein I and II	Insulation from PE in transgenic mouse	Palmiter *et al* 1993
Tyrosinase gene of mouse	DNase I hypersensitive site located at – 12 kb	Insulation from PE in transgenic mouse	Montoliu *et al* 1996
β-globin locus of human	DNase I hypersensitive site 5	Insulation from PE in transgenic mouse	Grosveld *et al* 1987; Li and Stamatoyannopoulos 1994
Growth hormone locus of human	Set of DNase I hypersensitive sites at – 15 and – 35 kb	Insulation from PE in transgenic mouse	Jones *et al* 1995
SCS and SCS′	HSP70 locus of *Drosophila*	Insulation from PE in *Drosophila*	Kellum and Schedl 1991
		Enhancer blocker in *Drosophila*	Kellum and Schedl 1992; Vazquez and Schedl 1994; Cai and Levine 1995
		Enhancer blocker in human T cell line	Zhong and Krangel 1997
BEAF32 targets/ CGATA motif of SCS′	Many sites in *Drosophila* genome	Insulation from PE in *Drosophila*	Cuvier *et al* 1998
Reiterated su(HW) binding sites	Gypsy retrotransposon and, possibly, at many other loci of *Drosophila* genome	Enhancer blocker in Drosophila	Geyer and Corces 1992; Cai and Levine 1995
		PRE blocker in *Drosophila*	Sigrist and Pirrotta 1997; Mallin *et al* 1998
		Insulation from PE in *Drosophila*	Roseman *et al* 1993
		Insulation of DNA replication origin from PE	Lu and Tower 1997
BX-C of *Drosophila*			
Mcp	A set of DNase I hypersensitive sites between *iab-4* and *iab-5*	Phenotype-A4 → A5 homeotic transformation	Karch *et al* 1994
Fab-6	A set of DNase I hypersensitive sites between *iab-5* and *iab-6*	Indirect deduction of A5 → A6 homeotic transformation phenotype	Mihaly 1998
Fab-7	A set of DNase I hypersensitive sites between *iab-6* and *iab-7*	Phenotype-A6 → A7 homeotic transformation	Gyurkovics *et al* 1990; Galloni *et al* 1993; Mihaly *et al* 1997

Table 4. (*Contd.*)

Boundary	Location	Assay	Reference
		Enhancer blocker	Hagstrom *et al* 1996; Zhou *et al* 1996
Fab-8	A set of DNase I hypersensitive sites between *iab-7* and *iab-8*	Phenotype-A7 → A8 homeotic transformation	Mihaly 1998
		Enhancer blocker	Mihaly *et al* 1998a; Barges *et al* 1998; Shanower *et al* 1998; Zhou *et al* 1999
ARS-1 element of yeast	Contains a SAR that can bind to plant nuclear scaffolds *in vitro*	Insulation from PE in stably transformed tobacco cell lines	Allen *et al* 1993
Boundaries of the HMR locus of yeast	Flank the HMR domain and contain TY1 LTR	Prevent the spread of silenced chromatin at the HMR locus and telomeric PE	Donze *et al* 1999
STAR	Subtelomeric regions of yeast	Prevent the spread of silenced chromatin at the HML locus and telomeric PE	Fourel *et al* 1999
UAS_{rpg}	Upstream of ribosomal protein gene TEF2 of yeast	Silencer blocker	Bi and Broach 1999
sns (silencing nucleo-protein structure)	3′ end of sea urchin early H2A histone gene	Enhancer blocker in sea urchin and human cell lines HeLa and U-2 OS	Palła *et al* 1997
RO (repeat organizer)	Within the intergenic spacer of *Xenopus* rRNA genes	Specialized insulator that works only in its normal position and orientation in *Xenopus*	Robinett *et al* 1997
BEAD-1 (blocking element alpha/ delta-1)	Between human TCR α and δ gene segments	Enhancer blocker in human T cell line	Zhong and Krangel 1997
3′ flanking region of human CD2 gene	A set of DNase I hypersensitive sites in the 3′-flanking region	Insulation from PE in transgenic mouse	Greaves *et al* 1989

The biochemical assays that are used to define MAR/SAR do not exclude the possibility that the interaction of DNA with the matrix is not altered during the experiment. In addition, transcription and replication processes may interfere with the matrix attachment although such artifacts can be avoided by using cells that are fully inactive for replication or transcription. Recently, however, more physiological and mild biochemical procedures have been employed to identify MARs. Furthermore, some of the identified MARs have been tested in the transgenic approach for insulating properties. While some MARs harbour boundary property when tested in other assays, see table 4 (Stief *et al* 1989; Phi-Van *et al* 1990; Schoffl *et al* 1993), many of them do not (Poljak *et al* 1994). Any analysis must, however, take into account that all MARs may not have similar properties at ectopic locations and in different contexts of adjoining regulatory elements as is the case in transgenic assays. There are several reports of MARs mapping within the regulatory regions or intron of genes (Gasser and Laemmli 1986; Oancea *et al* 1997). It is possible that there are different classes of MARs and some of them may not define boundary of a chromatin domain. Such MARs may function to bring a DNA region closer to the matrix to facilitate the access to regulatory proteins. Point mutations and/or *in situ* deletion in a particular MAR are necessary to understand its *in vivo* function. Such studies are yet to be reported.

3.2b *Locus control regions*: Locus control regions (LCR) are the DNA sequences that define a chromatin domain of independent regulatory environment. Among numerous LCRs that have been identified, the β-globin LCR has been studied most extensively. LCRs are characterized by a set of DNase I hypersensitive sites which contain binding sites for a variety of regulatory proteins. These elements also provide a copy number dependent and position independent expression of a

reporter gene. This and frequent mapping of MAR in LCRs (Jarman and Higgs 1988; Stief *et al* 1989; Zenk *et al* 1990) suggests that these elements define a functionally autonomous structural domain and that the boundary element is one of the indispensable components of an LCR. However, the mechanism by which the LCRs act and, in particular, how they contribute to the complex developmental regulation is unknown. Several extensive studies have suggested that LCRs are indispensable for appropriate execution of a developmental regulation programme, for example, of human β-globin locus in transgenic mouse (Grosveld *et al* 1987; Tewari *et al* 1996). A recent study, however, suggests that LCR may neither be necessary for the formation of DNase I hypersensitive sites nor for the developmental regulatory switches *ex vivo* in cell culture (Epner *et al* 1998).

3.2c *SCS/SCS′ elements*: SCS and SCS′ were originally identified as a set of DNase I hyper sensitive sites on both sides of the *Drosophila melanogaster* Hsp70 (heat-shock genes) at chromosomal map position 87A7 (Udvardy *et al* 1985). Since the 87A7 chromomere decondenses upon heat shock, forming a puff, it was suggested that this long distance effect is due to the accumulation of tortional stress as a consequence of high transcriptional activity in the topologically independent domain defined by the SCS and SCS′ elements. Indeed, SCS and SCS′ have been localized at the border of the puff and are sites of topoisomerase II activity (Udvardy *et al* 1986; Udvardy and Schedl 1993). Such observations suggest that these elements define the topologically independent structural and functional domain. SCS and SCS′ have been extensively studied using a variety of boundary assays. When a mini-white reporter gene construct is flanked by SCS and SCS′, the transgene is insulated from position effects. Finally, both elements have been shown to function as enhancer blockers in several different transgenic reporter systems (see table 4). Using an antibody raised against SCS′ binding protein (see below) in immunoprecipitation experiments with *Drosophila* genomic DNA, a class of chromatin boundary elements have been isolated (Cuvier *et al* 1998).

3.2d *Gypsy insulator*: Gypsy is a retrotransposon that was found associated with many mutations in *Drosophila* genes that have complex regulatory regions (Modolell *et al* 1983). Numerous studies have shown that in Gypsy insertional mutations the regulatory elements situated between the promoter and the insertion site are still operational while the ones located distally relative to the insertion site are inactivated. This suggests that Gypsy insertions behave as enhancer blockers or boundaries. Analysis of suppressors of these mutations identified the *su(Hw)* protein and a set of twelve binding sites for this protein in Gypsy, that are responsible for the enhancer blocking activity (Parkhurst *et al* 1988; Peifer and Bender 1988). Subsequent experiments with the yellow gene have shown that a 320 bp region of Gypsy that contains the twelve *su(Hw)* binding sites is sufficient to mimic the mutagenic effect of the Gypsy insertion and functions as boundary in insulator and enhancer blocking assays (Roseman *et al* 1993; Scott and Geyer 1995).

In transgene constructs where Gypsy insulator element is interposed between the promoter and a Polycomb response element (PRE, see below) (Sigrist and Pirrotta 1997), it can block the repressive effect of the PRE. This suggests that the blocking mechanism of Gypsy is such that it prevents both enhancers and silencers from acting across this boundary. Recently, MAR activity was mapped within Gypsy DNA (Nabirochkin *et al* 1998). Taken together, these observations suggest that Gypsy element and, perhaps, other genomic targets of *su(Hw)* protein are capable of creating a chromatin boundary likely by associating with the nuclear matrix. The *su(Hw)* antibody stains about 200 sites on the salivary gland chromosomes. Whether these targets of *su(Hw)* function as chromatin domain boundaries remains to be determined. It is interesting in this context that two recently discovered boundaries flanking the HMR locus of yeast contain LTR of TY1 transposon (Donze et al 1999). Further more, this TY1 LTR containing boundary function has been shown to be affected by mutation in chromosomal structural protein SMC1. These observations implicate transposable elements in structural/functional organization of genome in two distant species.

4. Chromatin domain boundaries in the bithorax complex

The BX-C of *Drosophila* contains three homeotic genes, *Ubx, abd-A* and *Abd-B* that are responsible for the identities of the parasegments that form the posterior half of the thorax and the abdomen. An unusually long regulatory region of 300 kb regulates *Ubx, abd-A* and *Abd-B* along the body axis to generate expression patterns unique to each parasegment (Peifer *et al* 1987). These complex expression patterns are set early in development by the segmentation genes which are only transiently expressed (Simon *et al* 1990; Qian *et al* 1991; Shimell *et al* 1994). Expression pattern of the homeotic genes is maintained by a different mechanism involving the *Polycomb* and *trithorax* group genes (Pc-G and trx-G) that some how imprint inactive or active state of the chromatin, respectively (Kennison and Tamkun 1988; Shearn 1989; Simon *et al* 1992; Pirrotta 1997; Paro *et al* 1998). The presence of homologues of these genes in evolutionarily distant species including mammals and plants suggests that the mechanisms of gene regulation based on the structure of chromatin are conserved.

The mutations in the regulatory region of the BX-C have helped to define the cis elements that respond to (i)

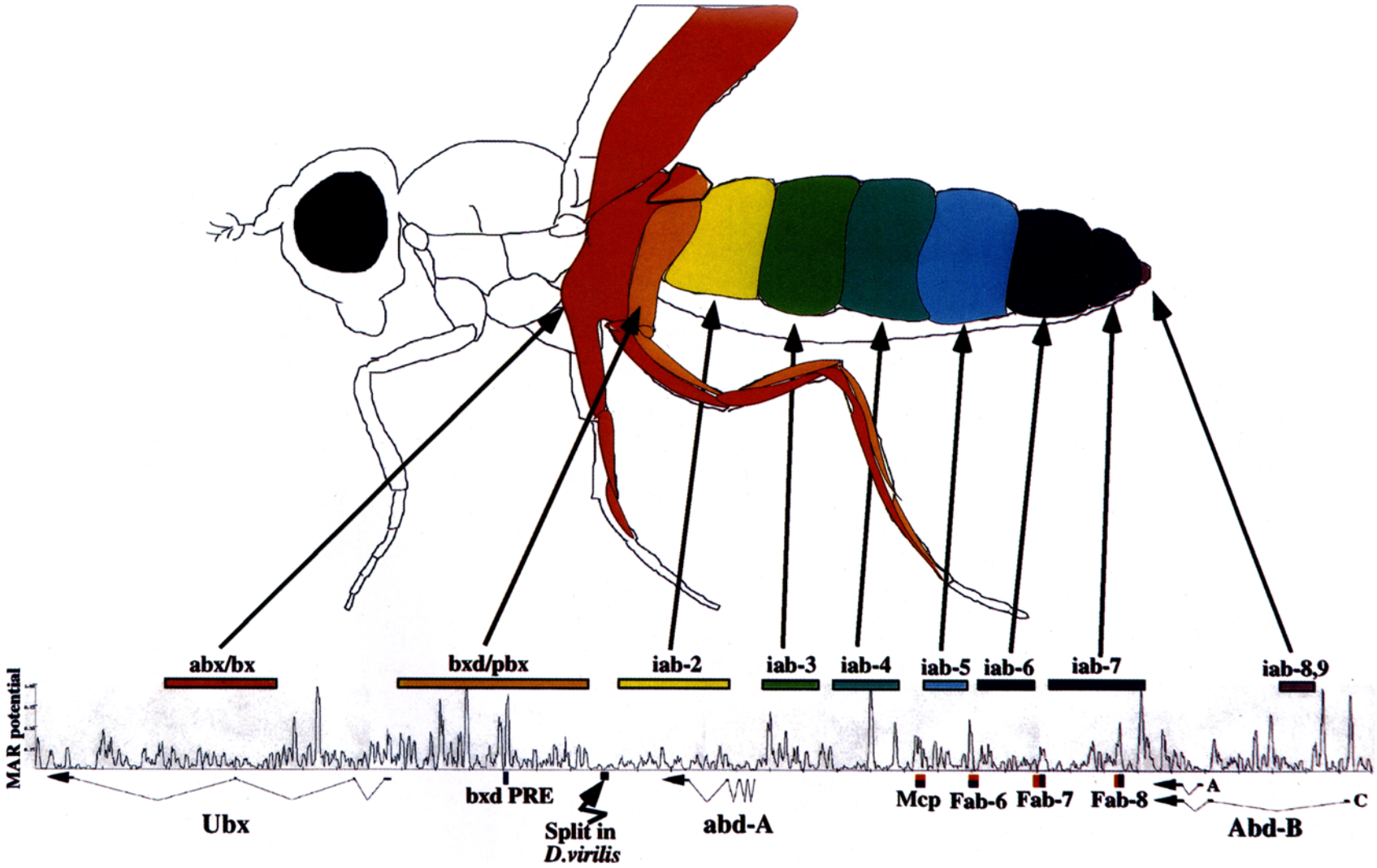

Figure 3. Organization of the bithorax complex of *D. melanogaster*. A plot of MAR potential (Singh *et al* 1997) along the entire BX-C spanning about 330 kb is shown. Transcription units of the three homeotic genes of the complex, *Ubx*, *abd-A* and *Abd-B*, are shown below the MAR plot. Regulatory elements, *abx/bx* to *iab-8,9*, defined by large number of mutations are drawn above the MAR plot. Mutations in a cis regulatory element give a recessive loss of function phenotype that affects only the corresponding body segment, shown by colour coding and arrows. On the contrary, mutations in the boundary elements shown below the MAR plot as red rectangles, *Mcp* to *Fab-8*, give a dominant gain of function phenotype. For example, a fly heterozygous for *Fab-7* mutation has no 6th but two 7th abdominal segments. Three PREs that have been mapped by functional assays (*iab-7* PRE and *iab-8* PRE adjacent to *Fab-7* and *Fab-8* boundaries, respectively, and *bxd* PRE shown as blue rectangles) show strong MAR potential, in particular the *bxd* PRE. The boundary and PRE functions are overlapping in case of *Mcp* and *Fab-6*. A pair of very strong MARs are seen at the end of the complex after *iab-8,9*. The region that has least MAR potential corresponds to the split in the complex in *D. virilis* shown as zigzag arrow. The precise site of this split was kindly provided by Prof. W Bender.

the initial setting of the chromatin structure by the segmentation genes, and (ii) the subsequent maintenance by the Pc-G and trx-G genes (Bender *et al* 1983; Karch *et al* 1985; Simon *et al* 1990; Muller and Bienz 1991; Qian *et al* 1991; Simon *et al* 1993; Chan *et al* 1994). It is now well established that the regulatory information to initiate and maintain segment specific expression pattern of BX-C genes is arranged in a linear fashion in the order of their expression along the anterior-posterior body axis (figure 3). It has been proposed that each parasegment-specific regulatory region corresponds to a chromatin domain. The regulatory region of the BX-C would, therefore, consist of an array of parasegment-specific regulatory domains separated by boundaries (Peifer *et al* 1987; Galloni *et al* 1993; McCall *et al* 1994). Two such boundary elements (*Mcp* and *Fab-7*) are defined on the basis of their dominant gain of function phenotypes associated with deletion mutants which fuse two adjacent parasegment-specific regulatory domains into one single functional unit (Gyurkovics *et al* 1990; Galloni *et al* 1993; Vazquez *et al* 1993; Karch *et al* 1994). Boundary function of the *Fab-7* element has been well established in a variety of studies (Hagstrom *et al* 1996; Zhou *et al* 1996; Mihaly *et al* 1997, 1998a). In collaboration with laboratories of Schedl and Gyurkovics, we have recently identified two other boundary elements of the BX-C viz., *Fab-6* and *Fab-8* (Mihaly 1998).

All the bithorax complex boundary elements are found to coincide with a set of DNase I hyper sensitive sites. Interestingly, polycomb response elements (PREs) – DNA sequences that recruit the *Polycomb* group of proteins – are found either as a distinct but adjacent DNase I hyper sensitive site or inseparable from the boundary elements. Extensive genetic and molecular analysis, identified sequence motifs and genetic loci that interact with these elements give us a glimpse of how chromatin domain boundaries might function (Hagstrom *et al* 1997; Mihaly *et al* 1997).

5. Common features among different chromatin domain boundaries

Boundary swapping experiments in our laboratory, wherein *Fab-7* boundary has been replaced by SCS or 12 reiterated *su(Hw)* binding sites, suggest that boundaries identified at different loci in *Drosophila* behave differently, although both of them can substitute for *Fab-7* in preventing adventitious interactions between adjacent regulatory domains (Mihaly *et al* 1998a; I Hogga and F Karch, personal communication). It is likely that while having some characteristics in common, individual chromatin domain boundaries may be associated with other regulatory elements. For example, the BX-C boundaries are associated with PREs and boundaries near the mammalian LCRs are associated with various regulatory elements. Different kinds of boundaries with variable regulatory properties, thus, appear to be a common feature of eukaryotic chromatin organization. If boundaries are close to the nuclear matrix, they might be conveniently placed in the vicinity of accessible regions of the genome. It is not surprising, in this regard, that boundary elements that define the domain of a gene activity are often juxtaposed to regulatory elements. Mapping of MARs in LCRs and the presence of YY1 binding sites in most PREs (see below) suggests that these elements might be interacting with the nuclear matrix (Robinson *et al* 1982; McNeil *et al* 1998).

DNA sequence comparison of various boundary elements has failed to identify any significant homology, except small conserved sequence motifs of unknown significance or stretch of AT rich regions (Vazquez *et al* 1993; Karch *et al* 1994). In contrast, most boundary elements can be mapped by a set of DNase I hypersensitive sites. This suggests that there are positioned nucleosomes and perhaps some secondary structural features that are shared among different boundaries. It seems likely that different boundaries contain binding sites for a subset of proteins and that characteristic of a boundary will depend upon the actual composition of its DNA protein complex.

In this view, small sequence motifs that interact directly with boundary binding proteins recruit other factors to establish a structure. Such sequence motifs, however, escape computer programs that hunt for the homologies and similarities. This is supported by the fact that there is a striking functional conservation among boundary elements isolated from a variety of systems and by different assays (see table 4). At present, we do not fully understand the structural basis of this functional conservation.

6. Proteins associated with chromatin domain boundaries

Nuclear matrix or skeleton is the major proteinaceous frame work for chromatin organization and likely to provide some of the protein components of chromatin domain boundaries. However, the nuclear matrix also serves to organize the structures that carryout transcription, splicing, replication, nucleolus and other architectural elements of the nucleus. It is, therefore, likely that most of the matrix proteins may not be directly involved in boundary formation. Methods used to prepare such a matrix also have been subject to criticism in the past as the preservation of the fine structure or protein (and DNA in the case MAR isolation) content is expected to be less than complete. Such problems have been considerably resolved by recent improvements in matrix preparation procedures (Jackson *et al* 1990a; Wan *et al* 1999). Preparations of chromatin free matrix structure

provides an insight into how the chromatin may be organized in the nucleus on a framework of proteins. The matrix network appears to be made of protein fibers of 10 nm thickness and several such fibers emanate from hundreds of junctions which may some times coincide with various sub-nuclear structures, viz., transcription, replication, RNA processing centers, nucleolus, etc. (Capco *et al* 1982; Jackson and Cook 1985; Hassan and Cook 1993; Hozak *et al* 1993, 1994).

Specificity of interaction of MAR with the matrix is thought to be mediated by many sequence motifs spread over several hundred nucleotides and the DNA binding proteins that recognize certain structural features in the minor groove of SAR DNA (Churchill and Travers 1991; Laemmli *et al* 1992). Several SAR/MAR binding proteins are listed in table 5. A special AT-rich sequence binding protein 1 (SATB1) was isolated by screening human cDNA expression library using MAR sequence as probe (Dickinson *et al* 1992). In addition to the MAR binding domain this protein also contains an atypical homeo-domain and two cut-like repeats (Dickinson *et al* 1997). SATB1, expressed predominantly in thymocytes, binds to minor groove of DNA with little contact to the bases, suggesting that the protein recognizes some secondary structural feature of DNA. Three other proteins with similar high affinity and MAR specific binding have been identified: nucleolin, a major nucleolar protein with multiple functions (Dickinson *et al* 1997), p114, isolated from breast carcinoma (Yanagisawa *et al* 1996) and Bright, a B-cell specific protein (Herrscher *et al* 1995). Other proteins known to bind MARs are Topoisomerase II (Adachi *et al* 1989) and lamin B1 (Luderus *et al* 1992). From the nuclear abundance of these proteins and their biochemical characteristics, it is suggested that they play an important part in defining the topologically independent domains of chromatin.

Boundary activity in Gypsy insulator is dependent on protein product of *su(Hw)* gene (see § 3.2d). Another gene identified initially as *mod(mdg4)* mutation is also indispensable for the boundary function of Gypsy element (Gerasimova *et al* 1995). These two proteins have been shown to interact genetically and physically with the Gypsy insulator (Gdula *et al* 1996; Gerasimova and Corces 1996, 1998). Mutations in the gene coding for *mod* behave like mutation in the *trithorax* group of genes suggesting a possible link between the boundaries mediated by the *su(Hw)* and the Pc-G and trx-G proteins (Dorn *et al* 1993; Gerasimova and Corces 1998). While the Gypsy is normally not part of the *Drosophila* genome, both *su(Hw)* and *mod* are known to bind to hundreds of sites on the salivary gland polytene chromosome. *mod* has also been isolated as an enhancer of PEV (Dorn *et al* 1993). It is not clear though how *mod* contributes to the boundary mechanism of Gypsy. It will be important to know the genomic targets of *su(Hw)* and *mod*, which may define a class of boundary elements.

Proteins binding to SCS and SCS′ elements have been identified. Boundary element associated factors, (BEAF) 32A and BEAF 32B have been shown to bind not only to the SCS′ site in the chromosome but also at hundreds of other sites (Zhao *et al* 1995; Hart *et al* 1997). No mutation in BEAF has been recovered so far. An SCS binding protein (SBP) has been identified recently and is found to be encoded by *zw5* (Gaszner *et al* 1999). Localization of SBP site on polytene chromosomes and the *zw5* phenotype suggests that this protein also interacts with many other sites in the genome and serves an essential function (Gaszner *et al* 1999; Udvardy 1999).

We have isolated proteins that recognize sequence motifs in *Fab-7* boundary DNA of the bithorax complex. At least one of these proteins is novel and binds to many sites on the salivary gland chromosome. We are in the process of identifying other proteins that bind to the boundary and/or PRE region of *Fab-7*. During these studies we also found GAGA factor to be interacting with both regions of *Fab-7* (see also Strutt *et al* 1997). GAGA is a uniformly expressed protein that has been implicated in trancriptional control and chromatin organization (Granok *et al* 1995). Recently the first DNA binding Pc-G gene, *pleiohomeotic* (*pho*), was cloned and found to be the fly homologue of the multifunctional factor YY1 (Brown *et al* 1998). We have found that all PREs have an extended YY1/PHO binding sites (Mihaly *et al* 1998b) and that these sequence motifs are important for *in vitro* binding of PHO as well as *in vivo* PRE activity (J Mihaly, R K Mishra and F Karch, unpublished result).

One general conclusion that we can draw from these studies is that boundary elements are built of several sequence motifs recognized by different proteins. A subset of these factors and additional sequence features, for example, the nucleosome positioning or DNA bending sequences, may be able to create a boundary structure. Additional regulatory elements when associated with the boundary elements may integrate a regulatory input into the boundary function. Some boundaries, therefore, may have unique or special properties.

7. Possible models for chromatin domain boundaries in the interphase nucleus

Since only a few chromatin domain boundary elements have been studied in sufficient details so far, it is difficult to understand the structural basis of how such elements function. The models explaining boundary function, therefore, remain very speculative figure 4. In this section we discuss various possibilities by synthesizing isolated observations in the context of what is expected from a boundary element and how this could fit in a structural framework.

Creation of topologically independent domains necessitates securing the two ends of the domain to matrix.

Table 5. Boundary/MAR interacting proteins.

Protein	Isolation/interaction	Comments	Reference
ARBP	Isolated from chicken using binding to MAR	Selectively and cooperatively binds to MARs, a component of nuclear network	von Kries *et al* 1991
		Homologous to rat MeCP2	Weitzel *et al* 1997
NMP1/YY1	*In vitro* binding	Homologous to *Drosophila* Pc-G protein PHO	Guo *et al* 1995; Brown *et al* 1998
	In situ immunofluorescence	Matrix targeting signal mapped	McNeil *et al* 1998
NMP2	*In vitro* binding	A variant of AML/PEBP2/runt domain protein	Merriman *et al* 1995; Lindenmuth *et al* 1997
MAR binding filament-like protein (MFP1)	*In vitro* binding	Contains a transmembrane domain, can discriminate between animal and plant MAR	Meier *et al* 1996
		DNA and non-MAR DNA fragments. Localizes to discrete domains at the nuclear envelope	Gindullis and Meier 1999
Boundary element associated factor (BEAF)	Purified from *Drosophila* cell culture using CGATA sequence motif of SCS′ as probe	Immunolocalization to hundreds of interbands and many puff boundaries, including SCS′, on polytene chromosomes	Zhao *et al* 1995
SCS binding protein (SBP)/zw^5	Expression library screen using SCS DNA as probe	SBP is encoded by the *zest-white* 5 gene, binds to SCS *in vivo*, mutations in zw^5 reduce enhancer blocking by the multimeric SBP binding sites	Gaszner *et al.* 1999
SATB1	Expression library screen using SAR DNA as probe	A tissue-specific MAR binding protein	Dickinson *et al* 1992
		Sequences bound to SATB1 *in vivo* are tightly associated with the nuclear matrix at the base of the chromatin loops	de Belle *et al* 1998
ScI/Topoisomerase II	Initially isolated as a component of nuclear scaffold	High abundance in matrix (~ 3 molecules/loop). Localized at the base of chromatin loops	Earnshaw and Heck 1985; Gasser *et al* 1986
		Preferential and cooperative MAR binding	Adachi *et al* 1989
SAF-A (hnRNP-U)	*In vitro* binding and *in vivo* cross-linking	Binds specifically to MAR and chromosomal DNA *in vivo*	Gohring and Fackelmayer 1997
		Couples splicing to MAR	Nayler *et al* 1998
Mutant p53	*In vitro* binding	This interaction with MAR is distinguishable from the DNA binding of wild type protein	Muller *et al* 1996
Histone H1	*In vitro* interaction	Binds MAR DNA cooperatively, possibly, to bring about chromatin condensation	Izaurralde *et al* 1989; Laemmli *et al* 1992
HMG-I/Y	*In vitro* interaction	Non cooperative binding	Reeves and Nissen 1990; Zhao *et al* 1993
Nucleolin	DNA affinity column purification	Preference for base unpairing region of MAR	Dickinson and Kohwi-Shigematsu 1995
Bright (B cell regulator of IgH transcription)	Isolated as IgH MAR binding protein	Requires a tetramerization domain for DNA binding which is also shared by SWI1 of SWI/SNF complex	Herrscher *et al* 1995
su(HW)	Isolated as suppressor of gypsy induced mutations	Binds to 100–200 sites on *Drosophila* polytene chromosomes, recognizes PyPuTTGCATACCPy sequence present 12 times in gypsy	Spana *et al* 1988

Table 5. (*Contd.*)

Protein	Isolation/interaction	Comments	Reference
mod(Mdg4)	Isolated as an interactor of *su(HW)*	Localizes at about 400 sites on *Drosophila* polytene chromosome	Gerasimova *et al* 1995; Gerasimova and Corces 1998
		Isolated as Enhancer of PEV, $E(var)^{3-93D}$	Dorn *et al* 1993
GAGA factor	Purified from *Drosophila* nuclear extract, coded by *trl* gene	Shown to bind to *Fab-7* boundary region	Farkas *et al* 1994; Strutt *et al* 1997
		Shown to be involved in enhancer blocking.	Ohtsuki and Levine 1998
	Expression library screen and affinity column with *Fab-7* DNA	Shown to bind to *Fab-7* boundary region	R K Mishra and F Karch, unpublished

Several putative MAR binding proteins have been isolated (Razin *et al* 1981) and, in addition to SCI/topoisomerase II, several other scaffold proteins have been identified, for example, ScII, Lamin A, B, C (Lewis *et al* 1984). Their role in boundary function remains to be confirmed. In a recent report, mutation in SMC1 gene has been shown to affect the boundary element of the silenced HMR domain in yeast (Donze *et al* 1999).

Boundary elements may function in association with nuclear matrix through SAR/MAR like elements. In the next level of organization, boundaries may function to arrange chromosomal domains in nuclear compartments in a way that regions requiring similar regulatory environment are placed together. This may involve clustering of boundary elements to form chromatin granules and result into compartmentalization of chromosomal territories and the nucleus. Clustering of boundaries may take place along the nuclear matrix. However, it is possible that several regions of the chromosome may come together and remain associated if some components of the chromatin can interact to stabilize the structure. In such an organization the nuclear matrix may not be directly involved.

Association of BX-C boundaries with PRE and ability of the PREs to cluster together may suggest that the clustering/pairing of chromatin structure may initiate from one boundary and end at another one in cis, providing a structural basis for functional domains (see figure 3 and § 4 and 6). Among the proteins that interact with PREs is *pho*, which is a homologue of mammalian protein YY1. This homology is significant from nuclear organization point of view. YY1 is known to associate with nuclear matrix and high molecular weight complexes (Guo *et al* 1995). The region of YY1, which is responsible for this matrix association, has been mapped (McNeil *et al* 1998) and is at least in part conserved in the fly homologue. It is possible, therefore, that Pc-G proteins might function in cooperation with the nuclear matrix by sequestering the DNA 'tagged' for repression to the inactive compartments. Boundary elements may set the limits of such repression and hence provide an explanation why in the bithorax complex boundaries are in close proximity to PREs.

Matrix attachment, pairing, clustering and compartmentalization based mechanisms may not be mutually exclusive. On the contrary, we think that chromatin domain boundaries function in a way that includes several of these processes. Also, boundaries may vary in their properties depending on their constituents and genomic location. For example, some boundaries may function as such only in certain cell types. Putative boundaries that may demarcate heterochromatin and euchromatin junctions or those that include a functional origin of replication should be significantly different from those functioning only to define regulatory units of a gene or packaging of DNA. In a subset of boundary elements, binding sites for a variety of factors that can cooperate or antagonize among themselves, may provide regulatory switches capable of responding to signaling mechanisms.

Several MARs have been found to be associated with regulatory elements like promoters or enhancers (Gasser and Laemmli 1986; Oancea *et al* 1997). It has also been found that SCS′ element is associated with a promoter (Glover *et al* 1995) and recently the EST database of *Drosophila* shows that there are promoters within SCS (Avramova and Tikhonov 1999). Such observations have led to the suggestion that these boundary elements may not be neutral structural elements and that their enhancer blocking activity may be due to the titration of enhancer function by the promoter within the boundary elements (Geyer 1997; Avramova and Tikhonov 1999). This, however, fails to explain why boundary elements have to be between the enhancer and the promoter to function as enhancer blockers and how boundary elements block transgenes from repressive effects. Finally, the genetic analysis of boundary elements of *Drosophila* BX-C also discounts such models since the phenotype of boundary deletion mutations can not be explained by the assumption that these deletions remove promoter like elements (see figure 3 and § 4 and 5). Cohabitation of boundary elements with regulatory elements suggest that proximity to boundary is not refractory to transcription, on the contrary it may be advantageous in the genomic context as the presence of a boundary will impose directionality (by

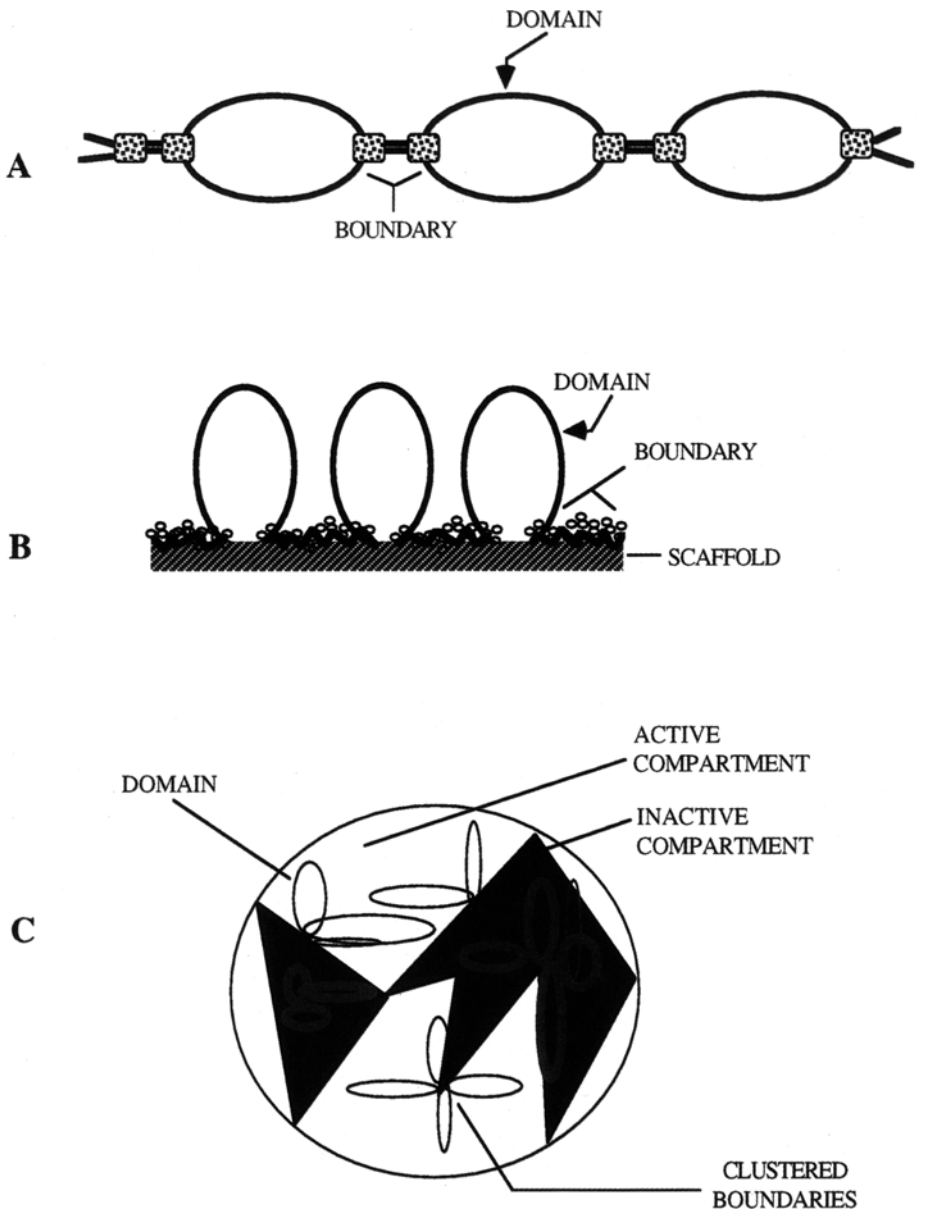

Figure 4. Chromatin domains and boundaries in the organization of eukaryotic genome. Three cartoons show how interactions among chromatin domain boundaries and nuclear matrix may provide structural basis to define functional chromatin domains. (**A**) Homologous chromosomes are shown paired in such a way that regulatory elements including boundaries from the two chromosomes, shown as dotted rectangle, are in contact. It is known that homologous chromosomes of *Drosophila* are paired during interphase. However, such a pairing is absent in most cell types of other eukaryotes. (**B**) Boundary elements are attached to the matrix/scaffold bringing the regulatory sequences into a nuclear compartment. Group of small circles represent structural proteins that mediate interaction of boundaries with the nuclear matrix. (**C**) Several boundaries are shown to cluster together and, thereby, bringing the associated domains to active or inactive compartments, shown as clear and shaded sectors, respectively. Chromatin fiber in the inactive shaded sector is drawn thicker than the one in active compartment to suggest that in addition to being in different compartments active and inactive chromatin may be packed differently.

preventing the activity in one direction beyond the boundary element) and may be a mechanism to bring such regulatory elements to a particular chromatin context or compartment.

8. Boundaries, PREs and *Hox* gene clustering

We have reviewed the work that argues for a role of chromatin domain boundary elements associated with PREs in the regulation of the bithorax complex. One intriguing feature of all *Hox* genes is that they are clustered together and the order in which they lie in the chromosome corresponds to the order in which they are expressed along the anterior-posterior body axis (see figure 3). This is referred to as the principle of spatial colinearity. In case of vertebrates, *Hox* genes also follow the principle of temporal colinearity, that is, their organization reflects the temporal sequence in which they begin to be expressed during early development. It has been proposed that mechanisms that regulate *Hox* genes restricts them to such genomic organization (Duboule 1998). It is conceivable that the PREs/boundaries cluster together along the nuclear matrix in an "inactive compartment" and are sequentially released for activation. A critical combination of regulatory proteins could allow only one of the PREs to come off the inactive compartment. If a PRE from the middle of the silent complex dissociates, it will have to trade off two flanking PRE interactions in cis. This could ensure a sequential activation of the complex. In a recent report, DNA region required for the colinear expression of *HoxD* genes has been identified which seems to function by suppressing ectopic or premature transcription (Kondo and Duboule 1999). It remains to be seen if this DNA contains PREs or if there is a different/additional negative regulatory mechanism at work here.

If the organization of *Hox* complexes is conserved due to a mechanism that regulates them, at least some of the regulatory elements should also be conserved. Indeed, a boundary like 'relay element' has been suggested to exist between *Hoxd-13* and *Hoxd-12* in mouse (Kondo *et al* 1998). Also, like in *Drosophila*, *Polycomb* group genes and homeotic phenotype caused by mutations in these genes have been reported in many species (Singh *et al* 1991; Alkema *et al* 1997; Goodrich *et al* 1997; Gould 1997; Laible *et al* 1997; Grossniklaus *et al* 1998; Stankunas *et al* 1998). While these studies suggest that the colinearity rule of the *Hox* gene complexes, first discovered in *Drosophila* (Lewis 1978, 1985), may be a general one, subsequent studies have shown that there can be exception to this rule. While some insects have their *Hox* genes in single cluster, others have the complex split into two – between Ant-C and BX-C in *D. melanogaster* and *bxd/pbx* and *iab-2* in *Drosophila virilis* (Von Allmen *et al* 1996). It is interesting though that MAR prediction in the BX-C (see figure 3) shows two sets of strong candidates, one near *iab-8,9* and the other one near or within *bxd/pbx*, suggesting that the two sites may be able to anchor the complex to implement colinearity rule. That the *Hox* gene regulation is more complicated is suggested by strong transvection reported in the BX-C locus (Lewis 1954; Babu *et al* 1987; Mathog 1990; Martinez-Laborda *et al* 1992; Hendrickson and Sakonju 1995; Hopmann *et al* 1995; Gemkow *et al* 1998; Sipos *et al* 1998) and long range interaction of PRE bearing transgenes even when located on different chromosomes (Sigrist and Pirrotta 1997). Clearly, further studies are required to formulate a satisfactory model that can explain the chromatin organization and regulation of homeotic gene complexes.

9. Concluding remarks

It is becoming increasingly clear that organization of eukaryotic chromatin is intimately linked with the regulation of the genetic information. Domain boundaries that subdivide genome into functional units have been isolated from different species and show remarkable functional conservation when tested at ectopic locations or in heterologous systems. Boundaries identified thus far do not show any significant overall sequence homology. However, it seems likely that small sequence motifs, for example, binding sites for interacting proteins, may be shared by several boundaries. Analysis of such sequence motifs within boundary elements and proteins that interact with the boundary DNA has just begun to reveal the molecular basis of boundary functions. Finally, association of boundaries with regulatory functions involved in development such as PREs and LCRs suggests that chromatin domain boundaries may also be targets of developmental regulatory pathways.

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